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ANTIOXIDANT ACTIVITIES OF HYDROLYSATES ORIGINATED FROM SOYBEAN AND SOY MILK RESIDUE

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ABSTRACT

Soybean (*Glycine max*) and soy milk residue (okara) are protein-rich materials. Soybean possesses the highest protein content among different types of beans (protein content of soybean varies from 40–42 %). Soy milk residue, a by-product of the soy milk manufacturing industry, contains approximately 27 % protein (by dry weight). A number of recent studies have investigated the improvement of functional properties of protein contained in soybean and okara by fermentation or by the use of proteolytic enzymes. The aim of this study was to evaluate the antioxidant activities of soybean and okara hydrolysates obtained by the fermentation with *Aspergillus oryzae* or by using proteolytic enzymes (neutrase and flavourzyme). DPPH radical scavenging assay was used to determine the antioxidant activities of hydrolysates. The concentration of peptides required to scavenge DPPH radical by 50 % (IC_{50} value) was used to evaluate the antioxidant activity of peptides produced obtained from hydrolysates. The results showed that when fermented with *A. oryzae*, the okara hydrolysate had higher antioxidant activity than the soybean hydrolysate, with IC_{50} values of 0.447 mg/ml and 3.95 mg/ml, respectively. The hydrolyzed okara obtained from hydrolysis using Neutrase had higher antioxidant activity than the one obtained from hydrolysis using Flavourzyme, with IC_{50} values of 0.200 mg/ml and 0.407 mg/ml, respectively. Different peptide fractions obtained from the hydrolysates using cut-off membrane (10 kDa, 3 kDa and 1 kDa) possessed different antioxidant activities. The < 1 kDa peptide fraction exhibited the highest antioxidant activity with an IC_{50} value of 0.158 mg/ml.

Keywords: antioxidant activity, soybean hydrolysate, soy milk residue, *Aspergillus oryzae*, protease.

1. INTRODUCTION

Antioxidants play an important role in preserving food and preventing oxidation of fat. Antioxidants in food also helps protect consumers from harmful oxidizing agents such as toxins. The use of synthetic antioxidants is often harmful to health [1]. Biologically active peptides are very important natural antioxidants [2]. Many studies have shown the possibility to separate biologically active peptides from soy protein [3] or hydrolyzed soybean. Peptides that possess

antioxidant activities often consist of alanine, glycine, tyrosine, phenylalanine, serine and aspartic [4]. Soy milk residue (okara) is a by-product of the soy milk industry. Raw okara contains up to 25.4–28.4 % protein (dry matter) in insoluble form. Soybean residue contains 27% protein of dry matter [5] and consists of amino acids such as cysteine, methionine, valine, tyrosine, threonine, histidine and glycine [6]. These biologically active peptides can be obtained from protein fragments using proteases in the digestive system, microbial or plant enzymes or fermentation processes [7].

This study investigated the antioxidant activities of protein hydrolysates of soybean and soy milk residue obtained by fermentation with *Aspergillus oryzae* or by the use of proteolytic enzymes (Neutrase and Flavourzyme).

2. MATERIALS AND METHODS

2.1. Materials

The substrates used for hydrolysis in this study were soy milk residue (donated by Vietnam Soya Products Company) and a Vietnamese soybean variety. *Aspergillus oryzae* strains A1 and A2 were obtained from the Fungal Stock Culture Collection of the Department of Biotechnology, School of Biotechnology and Food Technology, Hanoi University of Science and Technology. Flavourzyme (originated from *Aspergillus orryzae*) and Neutrase (originated from *Bacillus amyloliquefaciens*) were purchased from Novozymes, Denmark. BHA (butylated hydroxyanisole), DPPH (2,2-diphenyl-1-picrylhydrazyl) and OPA (o-phthaldialdehyde) were purchased from Sigma, Aldrich.

2.2. Methods

2.2.1. Technological methods

Enzymatic hydrolysis procedure: 30 g of okara were added to 240 ml of distilled water. The mixture was then sterilized and undergone hydrolysis. The enzyme was added to the mixture in an enzyme: substrate ratio of 0.8 % (by dry weight). The temperature was maintained at 50 ^{o}C for 6 hours. Two stirring modes were tested during hydrolysis, in Hydrolysis mode 1 the frequency was 1 minute stirring after every 1 hour, in Hydrolysis mode 2 the frequency was 1 minute stirring after every 30 minutes.

Fermentation and hydrolysation with Aspergillus oryzae A1 and Aspergillus oryzae A2: 25 g of the sterilized substrate (okara or soybean) were inoculated with *Aspergillus oryzae* spores and then kept at 30 ^{o}C for 36 hours. When the spores grew into molds, the culture was filled with warm water in a ratio of 1:4 (w/w) and incubated at 55 ^{o}C for 48 hours.

Hydrolysate extraction method: Hydrolysates were prepared by the method described in [8] with minor modifications. 25 g of hydrolyzed material (okara or soybean) were added to 25 ml of distilled water and vortexed for 30 seconds. The obtained mixture was boiled for 3 minutes for enzymes deactivation. After boiling, sonication was carried out for 15 minutes, followed by centrifugation at 6000 rpm for 20 minutes to obtain the first fraction. The residue was washed by adding 10 ml of distilled water and centrifuged again to obtain the second fraction. The two fractions were collected, carefully mixed, filtered and used as extracts with antioxidant activity.

Peptides fractionation method: Peptides with antioxidant activity were fractionated through cut-off membrane following the method described in [9, 10] with some modifications.

First, the extracts were filtered through a 0.2 µm membrane then through 10 kDa, 3 kDa and 1 kDa cut-off membranes MWCO (Merck Millipore). All the obtained peptide fractions were used for analytical purposes.

2.2.2. Analytical methods

Determination of peptide content by OPA assay

OPA solution: 50 ml of OPA solution contains 25 ml sodium tetraborate 100 mM; 2.5 ml SDS 20% (w/w); 40 mg OPA (dissolved in 1 ml methanol); 100 µl of β-mercaptoethanol and 21.4 ml of distilled water.

OPA assay: 2 ml of OPA solution was added to 50 ml of extract from hydrolyzed material (sample solution) and mixed for 2 minutes. The optical absorption of the solution after the reaction was measured at 340 nm with quartz cuvette. The peptide content was calculated based on L-glutathione (reduction) calibration curve and expressed by mg of peptide per 1 gram of hydrolyzed material.

Determination of antioxidant activity by DPPH radical scavenging assay

The antioxidant activities of the hydrolysates and the peptide fractions extracted from the hydrolysates were determined by the DPPH radical scavenging assay described by Samruan et al. (2012) [11] with minor modifications.

a) 62.5 µM DPPH solution dissolved in methanol (100 ml) was prepared.

b) An amount of the hydrolyzed solution (60 µl or 150 µl) was added to a 50 ml fancol tube and dried at 90 °C for 30 minutes. The residue was added with 0.1 ml of methanol, shaken for 1 minute at room temperature, then added with 1.9 ml of DPPH solution, shaken and left to stand for 10 minutes. The color of test sample was measured at 515 nm using a colorimeter. The blank sample consisted of 0.1 ml of methanol and 1.9 ml of DPPH solution. Each determination was carried out in triplicate and the result was expressed as an average of three measurements with standard deviation. The inhibition concentration (IC) value was calculated based on the BHA standard curve.

The IC value is calculated by the formula: $\%IC = [(A_{blank} - A_{sample}) / A_{blank}] \times 100\%$

Where: A_{blank} - the absorbance of the blank sample.

A_{sample} - the absorbance of the test sample.

The IC_{50} value is calculated by the formula: $IC_{50} = (C \times 50\ \%)/y$, mg/ml

where: C - the peptide content of the sample (mg/ml), determined by the OPA assay;

y - the DPPH scavenging activity of the sample (%), that was calculated by using BHA as standards curve: $y = -181{,}59x + 99{,}907$; $R^2 = 1$, x was defined as the absorbance measured at 515 nm.

3. RESULTS AND DISCUSSION

3.1. The effect of microbial strains on the antioxidant activities of hydrolysates

Two strains of *A. oryzae* A1 and *A.oryzae* A2 were used to hydrolyze soybean and soy milk residue under the same conditions (temperature, incubation time, pH and substrate:water ratio).

The antioxidant activities of extracts and the peptide contents (expressed by mg per 1 g of hydrolyzed material) were presented in Table 1.

Table 1. Antioxidant activities of hydrolysates obtained from incubation with *A. Oryzae*.

Substrate	**Soy milk residue**		**Soybean**	
Strains of *A. oryzae*	**A1**	**A2**	**A1**	**A2**
Peptide content (mg/g)	33.964 ± 0.024	19.323 ± 0.046	84.626 ± 0.046	82.932 ± 0.055
IC_{50} (mg/ml)	0.447 ± 0.004	1.003 ± 0.005	3.950 ± 0.003	10.525 ± 0.004

It is clear from the above results that strain A1 produced hydrolysates with higher antioxidant activities than strain A2 for both substrates (soybean and okara).

3.2. The effect of heat treatment on the antioxidant activities of hydrolysates produced by using *A. oryzae*

In order to evaluate the effect of heat treatment on the antioxidant activities of hydrolysates, a series of experiments were carried out with soybean incubated with *Aspergillus oryzae*. Soybean was inoculated with *A. oryzae* for 48 hours at 55 ^{o}C. The hydrolyzed soybean was sterilized at 121 ^{o}C for 20 minutes. The peptide contents of extracts from sterilized samples are compared with untreated samples. The results are presented in Table 2.

Table 2. The effect of heat treatment on the antioxidant activities of hydrolysates.

Strains	***A. oryzae* A1**		***A. oryzae* A2**	
Mode of treatment	**No- heat treatment**	**Heat treatment**	**No- heat treatment**	**Heat treatment**
Peptide content (mg/g)	84.626 ± 0.046	195.396 ± 0.103	82.932 ± 0.055	265.373 ± 0.098
IC_{50} (mg/ml)	3.950 ± 0.003	7.760 ± 0.007	10.525 ± 0.004	14.714 ± 0.005

The results showed that when treated at 121 ^{o}C for 20 minutes, the peptide concentrations in samples increased by 2 ÷ 3 times compared with the untreated samples. This can be explained by the proteolysis of protein molecules into peptides at high temperature. However, the antioxidant activities of treated samples were lowered than that of untreated samples. Thus, the high-temperature treatment (sterilization) negatively affects the antioxidant activity of the product.

3.3. The effect of the type of enzyme and hydrolysis conditions on antioxidant activity of okara hydrolysates

Hydrolysis of okara was carried out with Neutrase and Flavourzyme in two different modes: Hydrolysis mode 1 and Hydrolysis mode 2. The hydrolysis conditions in the two modes are the same: the substrate:water ratio was 1:8, the temperature was 50 ^{o}C, the hydrolysis

duration was 6 hours, the enzyme:substrate ratio was 0.8 % of mass. The only different thing was the stirring mode as discribed above in 2.2.1 for enzymatic hydrolysis procedure. Antioxidant activities of okara hydrolysates depend on the used enzyme type and hydrolysis conditions, that is presented in Table 3.

Table 3. Effect of enzyme type and hydrolysis conditions on antioxidant activities of okara hydrolysates.

Enzyme type	**Non-hydrolysis (No enzyme addition)**	**Neutrase**		**Flavourzyme**	
Mode of hydrolysis		**Mode 1**	**Mode 2**	**Mode 1**	**Mode 2**
Peptide content (mg/g)	1.315 ± 0.016	1.999 ± 0.014	2.427 ± 0.017	2.881 ± 0.021	7.351 ± 0.018
IC_{50} (mg/ml)	1.556 ± 0.012	0.200 ± 0.009	0.242 ± 0.007	0.407 ± 0.007	0.829 ± 0.008

The above results indicated that the non-hydrolyzed okara has lower peptide content and higher IC_{50} than the hydrolyzed okara. The hydrolyzed product by Neutrase had higher antioxidant activity than the hydrolyzed product by Flavourzyme. Hydrolysates obtained from Hydrolysis mode 1 was better than the ones obtained from Hydrolysis mode 2 in terms of antioxidant activity. A study by Yokomizo et al. also showed that the soy milk residue hydrolysate obtained using protease N (originated from *Bacillus subtilis*) exhibited higher antioxidant activity than the one obtained using protease A (originated *Aspergillus oryzae)* [4]. Different properties of proteolytic enzymes originated from *Bacillus* and *Aspergillus* strains might be an explanation for the yield of peptides with different antioxidant activities.

3.4. Antioxidant activities of different peptide fractions

The extracts of hydrolyzed okara by Neutrase were separated into peptide fractions of different sizes by cut-off membranes. The antioxidant activity of each fraction was determined. The obtained results were shown in Figure 1 and Figure 2.

Hydrolyzed samples obtained from the use of Neutrase had significantly higher antioxidant activity than non-hydrolyzed samples. The IC_{50} values of peptide fraction with the size < 0.2 µm were 1.5 mg/ml and 1.077 mg/ml, respectively (Figure 1).

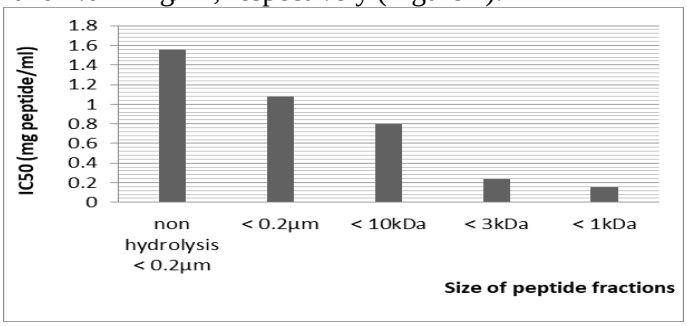

Figure 1. Antioxidant activities of peptide fractions from okara's hydrolysate and non hydrolyzed sample.

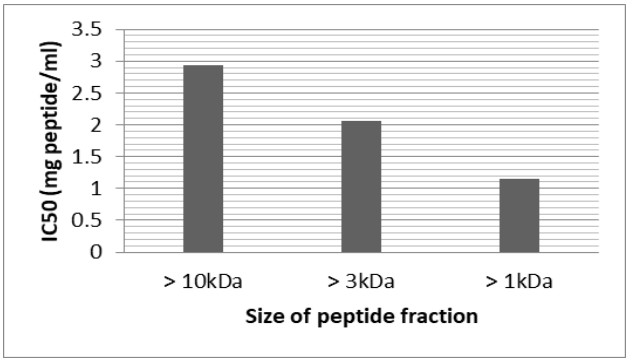

Figure 2. Antioxidant activities of peptide fractions in retentate of the cut-off ultrafiltration.

IC_{50} values of the < 3 kDa fraction and the < 10 kDa fraction were 0.235 mg/ml and 0.795 mg/ml, respectively. Thus, the < 3 kDa fraction had higher antioxidant activity than the fraction with size < 10 kDa. The < 3 kDa fraction consisted of only peptide fractions that had a size of < 3 kDa, while the < 10 kDa fraction consisted of peptide fractions that had a size of < 3 kDa and peptide fractions that had a size between 3 kDa and 10 kDa. This indicated that peptide fractions that have a size of < 3 kDa had higher antioxidant activity than peptide fractions that had a size of > 3 kDa. This is similar to results reported by Zou et al. [13] and Abu-Salem et al. [14]. The < 1 kDa peptide fraction had the highest antioxidant activity and the smallest IC_{50} value of 0.158 mg peptide/ml. This peptide fraction might consist of peptide segments with 3÷5 amino acids. According to Bougatef et.al. [15], a tri-peptide Leu-His-Tyr was fractionated from enzymatic hydrolysate of fish had a DPPH scavenging activity of 63 ± 1.57% at a concentration of 150 µg/ml and an IC_{50} value of 0.119 mg/ml. In another study on the antioxidant activity of protein hydrolysate of bluefin leatherjacket (Navodon septentrionalis) skin, three antioxidant peptides were extracted: Gly-Ser-Gly-Gly-Leu (GSGGL, BSP-A), Gly-Pro-Gly-Gly-Phe-Ile (GPGGFI, BSP-B), and Phe-Ile-Gly-Pro (FIGP, BSP-C) with molecular weights of 389.41 Da, 546.63 Da and 432.52 Da, respectively. Among these peptides, BSP-C exhibited the DPPH highest scavenging activity (with an IC_{50} value of 0.118 mg/ml) [16]. When compared with BHA and Vitamin C in terms of antioxidant activity, the < 1 kDa fraction had lower DPPH scavenging activity by 6.32 and 4.05 times, respectively (IC_{50} of BHA was 0.025 mg/ml; IC_{50} of Vitamin C – 0.039 mg/ml [11]); but higher antioxidant activity of Butylated hydroxytoluene (BHT showed IC_{50} value 0.241 mg/ml [11]).

4. CONCLUSIONS

The results of the study showed that the hydrolysis of okara by Neutrase gave product with higher antioxidant activity compared to non-hydrolysis when comparing IC_{50} values calculated as peptide concentration of 1.5 mg/ml and 1.077 mg/ml.

The okara's Neutrase hydrolysis gave higher antioxidant activity than Flavourzyme hydrolysis by comparing IC_{50} peptide values of 0.200 mg/ml and 0.407 mg/ml, respectively. The hydrolysis with stirring for 1 minute every 1 hour had higher effect in term of antioxidant activity than that with stirring for 1 minute every 30 minutes,

Both strains *A. oryzae* A1 and *A. oryzae* A2 had the ability to hydrolyze soy milk residue and soybean and turn protein molecules into peptides that possess antioxidant activity. The strain A1 was better the strain A2 (the corresponding IC_{50} values were 0.447 mg/ml and 1.003 mg/ml for okara hydrolysates, and 3.950 mg/ml and 10.525 mg/ml for soybean hydrolysates).

Different peptide fractions with different molecular sizes also have different antioxidant activities. The smaller the size of peptide fraction, the higher the antioxidant activity. The < 1 kDa peptide fraction exhibited the highest antioxidant activity with an IC_{50} value of 0.158 mg/ml. In further study, this peptide fraction will be identified for amino-acid components and characterized for amino-acid sequences which possess the antioxidant activity.

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TÓM TẮT

HOẠT TÍNH CHỐNG OXY HÓA CỦA SẢN PHẨM THỦY PHÂN TỪ ĐẬU NÀNH VÀ BÃ ĐẬU NÀNH

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Đậu nành *(Glycine max*) và bã đậu nành (okara) là những nguyên liệu giàu protein. Đậu nành có hàm lượng protein cao nhất trong các loại đậu (40–42 %). Bã đậu nành là một sản phẩm phụ từ ngành công nghiệp chế biến sữa đậu nành và đậu phụ, chứa khoảng 27 % protein (tính theo thành phần chất khô). Trong nghiên cứu này chúng tôi nghiên cứu so sánh hoạt tính chống oxy hóa của sản phẩm thủy phân protein đậu nành và bã đậu nành bằng 2 phương pháp: nuôi mốc *Aspergillus oryzae* và sử dụng chế phẩm enzyme protease neutrase và flavourzyme. Hoạt tính chống oxy hóa được xác định theo phương pháp thử khả năng bắt gốc tự do DPPH. Đã sử dụng giá trị IC_{50} (nồng độ dung dịch peptide có khả năng bắt gốc tự do DPPH 50 %) để đánh giá khả năng chống oxy hóa của peptide thu được sau khi thủy phân. Kết quả nghiên cứu cho thấy khi thủy phân bằng *Aspergillus oryzae*, hoạt tính chống oxy hóa của bã đậu nành lên men cao hơn so với hạt đậu nành lên men, với giá trị IC_{50} lần lượt là 0,447 mg/ml và 3,95 mg/ml. Sản phẩm bã đậu nành thủy phân bằng chế phẩm Neutrase có hoạt tính chống oxy hóa cao hơn chế

phẩm Flavourzyme với giá trị nồng độ peptide đạt IC_{50} lần lượt là 0,200 mg/ml và 0,407 mg/ml. Các phân đoạn peptide thu được từ dịch thủy phân với kích thước khác nhau (10 kDa, 3 kDa, 1 kDa) có hoạt tính chống oxy hóa khác nhau. Phân đoạn peptide < 1 kDa có hoạt tính chống oxy hóa cao nhất với IC_{50} peptide đạt được ở nồng độ 0,158 mg/ml.

Từ khóa: hoạt tính chống oxy hóa, sản phẩm thủy phân đậu nành, bã đậu nành, Aspergillus oryzae, protease.